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A molecular phylogenetics-based approach for identifying recent hepatitis C virus transmission events

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ABSTRACT

Improved surveillance methods are needed to better understand the current hepatitis C virus (HCV) disease burden and to monitor the impact of prevention and treatment interventions on HCV transmission dynamics. Sanger sequencing (HCV NS5B, HVR1 and Core-E1-HVR1) and phylogenetics were applied to samples from individuals diagnosed with HCV in British Columbia, Canada in 2011. This included individuals with two or three sequential samples collected <1 year apart. Patristic distances between sequential samples were used to set cutoffs to identify recent transmission clusters. Factors associated with transmission clustering were analyzed using logistic regression. From 618 individuals, 646 sequences were obtained. Depending on the cutoff used, 63 (10%) to 92 (15%) unique individuals were identified within transmission clusters of predicted recent origin. Clustered individuals were more likely to be <40 years old (Adjusted Odds Ratio (AOR) 2.12, 95% CI 1.21–3.73), infected with genotype 1a (AOR 6.60, 95% CI 1.98–41.0), and to be seroconverters with estimated infection duration of <1 year (AOR 3.13, 95% CI 1.29–7.36) or >1 year (AOR 2.19, 95% CI 1.22–3.97).

Conclusion: Systematic application of molecular phylogenetics may be used to enhance traditional surveillance methods through identification of recent transmission clusters.

1. Introduction

Hepatitis C virus (HCV) has infected between 115 and 185 million individuals globally (Gower et al., 2014; Mohd Hanafiah et al., 2013). As of December 2013, 73,500 (1.6%) individuals in British Columbia (BC) were diagnosed as anti-HCV positive (BC Centre for Disease Control, 2013). About two-thirds of anti-HCV positive individuals in BC are baby boomers born between 1945 and 1965. Most were infected decades ago and are at risk of developing progressive liver disease as they age. HCV-infected British Columbians have about a 5-fold increased risk of all-cause and 20-fold increased risk of liver-related mortality (Yu et al., 2013). HCV continues to be transmitted by people who inject drugs (PWID), who account for >80% of new infections in many countries (Hajarizadeh et al., 2013; Nelson et al., 2011). Reducing the population disease burden will require enhanced screening, engagement into care and treatment as well as the potential use of treatment-as-prevention to reduce transmission among PWID (Martin et al., 2013; Smith et al., 2012; Wiessing et al., 2014).

Informing effective public health interventions requires a clear understanding of the past and ongoing dynamics of viral epidemics. Phylogenetic trees constructed from viral sequences allow the historical relationships between infected individuals in a population to be modeled (Lam et al., 2010; Pybus and Rambaut, 2009;

Abbreviations: HCV, hepatitis C virus; PWID, people who inject drugs; BC, British Columbia; NS5B, non-structural protein-5B; E1, Envelope-1; HVR1, hypervariable region-1; cDNA, complementary DNA; RT-PCR, reverse transcriptase polymerase chain reaction; E2, Envelope-2; IU, international units; AOR, Adjusted Odds Ratio; CI, Confidence Interval; SD, Standard Deviation; OR, Odds Ratio; MSM, men who have sex with men.

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Volz et al., 2013). Distinct transmission clusters, i.e., groups of closely related infections, can be identified from phylogenies along with spatial, epidemiological and temporal information linked to their expansion (Grabowski and Redd, 2014; Hue et al., 2005; Lewis et al., 2008; van de Laar et al., 2009). The ability to quantify mutation rates enables recent transmission clusters to be identified and characteristics associated with high rates of transmission to be inferred (Aldous et al., 2012; Poon et al., 2014).

Phylogenetic cluster analyses are widespread in HIV research and have revealed that early/acute HIV infections play a major role in driving the epidemic and also that drug-resistant HIV variants can be transmitted and establish infection (Brenner et al., 2007; Kaye et al., 2008). HCV phylogenetic analyses have modeled the expansion of HCV throughout the 20th century in various global regions and have identified factors that likely contributed to the worldwide epidemic (Magiorkinis et al., 2009; Pybus et al., 2003, 2005; Tanaka et al., 2002). HCV phylogenetic cluster analyses have been used to confirm transmission events originating in health care settings and to investigate HCV clustering among HIV-infected individuals, men who have sex with men (MSM) and in PWID (Danta et al., 2007; Lanini et al., 2010; Oliveira et al., 2006; Sacks-Davis et al., 2012; Shemer-Avni et al., 2007; Urbanus et al., 2009; van de Laar et al., 2009). The criteria for defining transmission clusters differs for both HIV and HCV-based phylogenetics but when strict cutoffs are imposed, clusters that represent more recent transmission events can be identified. Tracking recent transmission clusters over time at a population level may be used to support HCV incidence estimations and to identify sub-populations at high risk of HCV transmission.

BC's centralized laboratory testing and consistent use of demographic identifiers enables identification of a subset of recent HCV infections by linking new anti-HCV positive to previous anti-HCV negative test results. However, most HCV-infected individuals have no prior testing history, precluding estimation of when transmission occurred. The aim of this study was to develop a method for identifying recent transmission clusters in a population. The HCV NS5B, core, E1 and HVR-1 regions were sequenced in a proportion of individuals who tested anti-HCV positive in BC in 2011. The genetic distances between sequential specimens from the same individuals collected less than one year apart were used to assign cutoffs to identify recent phylogenetic transmission clusters. This study provides a proof-of-principle for applying molecular phylogenetics to support traditional surveillance methods through identification of recent transmission clusters in a population.

2. Methods

2.1. Study population and design

The BC Public Health Microbiology and Reference Laboratory performs approximately 95% of HCV serological screening in BC (Yu et al., 2013). Automated linkage of anti-HCV positive tests with past results enables identification of seroconverters i.e., individuals who previously tested anti-HCV negative and are anti-HCV positive on subsequent testing. For seroconverters the estimated infection duration was defined as the time between the specimen collection date and the midpoint between an individual's last negative and first positive anti-HCV test.

Individuals who tested anti-HCV positive in BC in 2011 were eligible for assessment. Individuals were classified as: (1) *first time positives*: individuals who tested positive for the first time in 2011 and had no previous testing history; thus, their infection duration was unknown; (2) *past seroconverters*: individuals who seroconverted prior to 2011 but tested positive again in 2011 and thus had an estimated infection duration of >1 year; and (3) *recent seroconverters*: individuals who tested positive for the first time in 2011 and had an estimated infection duration of <1 year. The University of British Columbia Clinical Research Ethics Board approved this study.

2.2. HCV RNA testing and sequencing

HCV RNA was extracted from anti-HCV positive serum using the MagMAX™-96 Viral RNA Isolation kit (Life Technologies, Carlsbad, CA, USA). HCV viral loads were quantified using a semi-quantitative HCV RT-PCR assay (Meng and Li, 2010). Complementary DNA (cDNA) was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies). An 828 bp fragment containing a portion of the HCV polymerase (Non-Structural-5B; NS5B) and a 1514 bp fragment containing the HCV Core, Envelope-1, hypervariable region-1 (HVR1) and the beginning of Envelope-2 (E2) were amplified using Velocity DNA polymerase (Bioline, London, UK). Amplicons were purified using Agencourt Ampure XP beads (Beckman-Coulter, Mississauga, Ontario) and were Sanger sequenced using the BigDye Terminator Cycle Sequencing kit (Life Technologies). HCV RNA extraction, PCR, Sanger sequencing and thermal cycling conditions are described in detail in the Supplementary information.

2.3. Sequence analysis

Sequences were assembled and edited using Geneious v.6.1.7 (Biomatters, Aukland, New Zealand; http://www.geneious.com/). The NS5B sequences were trimmed to 650 bp. The Core-E2 sequences were trimmed to 920 bp and are referred to hereafter as Core-HVR1. Samples that had a partial but not a full Core-HVR1 sequence available were trimmed to 100 bp and contained the HVR1 region. Reference sequences obtained from the Los Alamos HCV sequence database (hcv.lanl.gov; Kuiken et al., 2005) (see Supplementary information) were included in the analysis. Sequences were aligned using MUSCLE (Edgar, 2004) and alignments were edited in Geneious. The program jModeltest-2.1.4 (Darriba et al., 2012) was used to determine the nucleotide substitution model that best described the aligned sequences, which was determined to be the General Time Reversible model with gamma distributed rate variation among sites and a proportion of invariable sites. Maximum likelihood phylogenetic trees were generated using FastTree2 (Price et al., 2010). Path-o-gen v.1.4 was used to estimate the best fitting root for the phylogenetic trees, which were visualized and annotated using FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Cluster analysis

Phylogenies included sequences from seroconverters who had sequential HCV positive samples collected <1 year apart. The patristic distances (i.e., genetic distance between two sequences in a phylogenetic tree measured in nucleotide substitutions per site) between these sequential samples represents HCV sequence evolution expected to occur within one year. Geneious software was used to determine the patristic distances between each pair of sequences in a phylogenetic tree, which include the pairwise intra-person (two sequences from one individual) and the within-genotype inter-person (two sequences from two different individuals) distances.

The intra-person distances were used to assign cutoffs that were applied to phylogenies using a custom python script (Poon et al., 2014). Sequences from different individuals with patristic distances falling below these cutoffs were interpreted as transmission clusters. The lower cutoff was set close to the maximum

observed intra-patient patristic distance (0.018 for NS5B, 0.03 for Core-HVR1 and 0.11 for HVR1). A second, higher cutoff was also assigned that excludes clusters between BC sequences and the Los Alamos reference sequences (0.020 for NS5B, 0.06 for Core-HVR1 and 0.15 for HVR1). A sensitivity analysis was performed using a third cutoff (0.022 for NS5B, 0.09 for Core-HVR1 and 0.19 for HVR1). Clusters within phylogenetic trees were manually examined; sequences from clusters that did not group together with branch support >80% were split into smaller phylogenetically linked clusters or excluded from the analysis. Clusters were visualized using Graphviz v.2.36 (Gansner and North, 1999).

To provide additional validation for the selected patristic distance cutoffs, six NS5B reference sequences were obtained from Genbank (Supplementary material) representing three pairs of confirmed transmission events (2 individuals/transmission) (Nakayama et al., 2005; Toda et al., 2009). The sampling intervals between each of the two individuals in the reported transmission pairs were between 2 weeks and 3 months. At least one individual from each transmission pair was diagnosed with acute HCV at the time of sampling i.e., transmission occurred approximately 6 months or less prior to sample collection. A phylogenetic and cluster analysis using these additional sequences (along with the BC sequences and Los Alamos reference sequences) was performed to determine the patristic distances between individuals in transmission pairs.

2.5. Statistical analysis

Logistic regression was used to identify factors hypothesized to be associated with recent infection, including age, sex, HCV genotype, seroconversion status, HIV status and geographic location (Jacka et al., 2014; Miller et al., 2002; Oliveira et al., 2009; Sacks-Davis et al., 2012). As 84% of sequences were genotypes 1a and 3a, the remaining genotypes were grouped together for analysis. Age was dichotomized to <40 vs. $\geqslant$40 years since unadjusted analysis demonstrated that individuals <30, 30–34 and 35–39 years were more likely to be in clusters than those >61 years (data not shown). In multivariate analyses, factors that were significant at $p < 0.20$ in the unadjusted analysis were considered as potential independent variables associated with clustering. For individuals with sequential samples, the first sample per individual was used. Analyses were performed in R, version 3.03 (R Core Team, 2013).

3. Results

3.1. HCV sequencing

In 2011, of 3356 individuals that tested anti-HCV positive in BC, 51% ($n = 1715$) were first-time anti-HCV positive (unknown infection duration), 38% ($n = 1267$) seroconverted between 1993 and 2010 (estimated infection duration >1 year), and 11% ($n = 374$) seroconverted in 2011 (132 had an estimated infection duration <1 year and 242 had an estimated infection duration >1 year).

For this study, 1546 of the 3356 anti-HCV positive samples were selected for analysis, including: (1) 897 randomly selected samples from the 1715 first time positive individuals; (2) 222 randomly selected samples from the 1267 seroconverters diagnosed prior to 2011 (many of whom are likely chronically infected), and (3) all available samples from 351 of 374 individuals who seroconverted in 2011 (283 had one sample and 68 had two or three sequential samples; total samples = 427) (Fig. 1A).

Of the 1546 samples selected, 1138 (74%) had detectable HCV RNA (>200 IU/ml), which yielded 646 sequences from 618 individuals (593 NS5B and 415 Core-HVR1) (Fig. 1A). For 52 additional samples, only a partial Core-HVR1 sequence was available; for these, the HVR1 sequence alone was examined and compared to all available HVR1 sequences ($n = 466$). Of the 68 individuals with sequential samples, sequences were obtained from 25 individuals. There were 363 samples that yielded sequences for all three regions (Fig. 1B). HCV RNA viral load of >20,000 IU/ml and being a seroconverter were associated with successful sequencing (Supplementary Table S1). Study subject characteristics are summarized in Table 1.

3.2. HCV phylogenetics

Phylogenies for NS5B, Core-HVR1 and HVR1 were constructed (Supplementary Figs. S1–S3). As expected, HCV sequences grouped together by genotype in both the NS5B and Core-HVR1 phylogenies. When HVR1 was examined alone, the majority of sequences also grouped by genotype, however there were several sequences that did not group with the predicted genotype. This likely reflects the high heterogeneity of HVR1 and the fact that many mutations in this region are driven by host immune pressure (Dowd et al., 2009; Liu et al., 2010). Also apparent from the phylogenetic trees is that the genotype 1a sequences form two large and distinct phylogenetic clusters.

3.3. Identification of HCV transmission clusters

Within each phylogeny, the patristic distances between pairs of intra-person sequential samples were determined and used to set cutoffs for identifying clusters as described in the Methods.

For NS5B, 593 sequences were obtained from 574 individuals; 17 individuals had sequential samples with a median (interquartile range) of 75 (21–161) days between sample collections. The inter-person patristic distances ranged from 0 to 0.35 (median 0.10) (Fig. 2A). Note that the presence of the bimodal hump in the distribution presented in Fig. 2A reflects the presence of the two genotype 1a phylogenetic groups. The intra-person patristic distances ranged from 0 to 0.017 (Fig. 2A). Of note, three sequential samples from one individual from 2009 and 2010 (23–454 days apart) also had patristic distances between 0 and 0.017. Applying the lower (0.018) and higher (0.020) cutoffs to the phylogeny identified 55 individuals (10%) in 25 clusters and 73 individuals (13%) in 31 clusters, respectively (Fig. 3A and Table 2).

To provide additional validation, an analysis of three confirmed transmission pairs using published NS5B sequences was performed (Nakayama et al., 2005; Toda et al., 2009). The three transmission pairs were found to cluster with patristic distances of 0.002, 0.006 and 0.01, respectively.

For Core-HVR1, 415 sequences were obtained from 396 individuals; 18 individuals had sequential samples with a median (interquartile range) of 71.5 (22.5–122) days between sample collections. The inter-person patristic distances ranged from 0.004 to 0.55 (median 0.22) (Fig. 2B). The intra-person patristic distances ranged from 0.0003 to 0.026 (Fig. 2B). Applying the lower (0.03) and higher (0.06) cutoffs to the phylogeny identified 24 individuals (6%) in 12 clusters and 48 individuals (12%) in 23 clusters, respectively (Fig. 3B and Table 2).

For HVR1, 466 sequences were obtained from 445 individuals; 20 individuals had sequential samples with a median (interquartile range) of 71.5 (21.5–117.8) days between sample collections. The inter-person patristic distances ranged from 0.01 to 2.91 (median 0.78) (Fig. 2C). The intra-person patristic distances ranged from 0 to 0.109 (Fig. 2C). Applying the lower (0.15) and higher (0.19) cutoffs to the phylogeny identified 19 individuals (4%) in 9 clusters and 31 individuals (7%) in 15 clusters respectively (Fig. 3C and Table 2).

When the sequences for all three regions were combined, 63 (10%; lower cutoffs) and 92 (15%; higher cutoffs) unique individuals were identified as belonging to clusters. In all, 27 individuals in 13

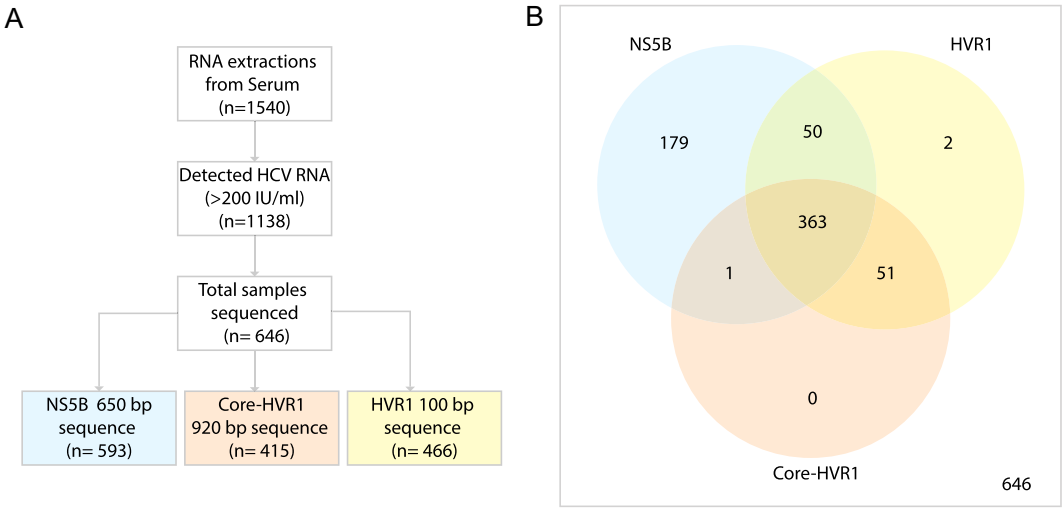

Fig. 1. Sequences obtained for HCV NS5B, Core-HVR1 and HVR1 regions. (A) Number of samples extracted, number samples with detectable HCV (>200 IU/ml), and number of sequences from each HCV genomic region. (B) Distribution of sequences for each of the HCV genomic regions.

Table 1
Characteristics of individuals with available HCV sequences ($n = 618$).

Characteristic	No. of individuals
Sex	
Female	202
Male	415
Unknown	1
Age	
Mean (SD)	46.2 (12.5)
<40 year	258
⩾40 year (baby boomers)	360
HCV genotype	
1a	393
1b	35
2a	22
2b	37
3a	124
Other	7
BC geographic region	
Interior	114
Fraser	207
Vancouver Coastal	140
Vancouver Island	82
Northern	60
Unknown	15
Test group	
Recent seroconverter[a]	52
Past seroconverter[b]	203
First-time HCV positive[c]	363
HIV co-infection	
Yes	16
No	436
Unknown	166

[a] Individuals with estimated infection duration <1 year.
[b] Individuals with estimated infection duration >1 year.
[c] Individuals with unknown infection duration.

clusters were identified within both the NS5B and HVR1 or Core-HVR1 regions (Fig. 3). Of note, 29 first time positive individuals with unknown infection duration were identified within clusters, suggesting that they were involved in recent transmission events.

3.4. Factors associated with transmission clusters

Factors associated with clustering were examined using the NS5B region, as this region has the greatest amount of data available. Using unadjusted analysis, factors associated with NS5B clusters identified using the higher cutoff (0.02) included age, HCV genotype 1a and 3a, and being a seroconverter (recent or past) (Supplementary Table S2). In adjusted analysis, age <40 years vs. ⩾40 years (Adjusted Odds Ratio (AOR) 2.12, 95% CI 1.21–3.73), genotype 1a infection vs. other genotypes (AOR 6.60, 95% CI 1.98–41.0), and being a recent seroconverter vs. first time positive (AOR 3.13, 95% CI 1.29–7.36) or a past seroconverter vs. first time positive (AOR 2.19, 95% CI 1.22–3.97) were independently associated with clustering (Table 3 and Supplementary Table S2). Unadjusted analysis of NS5B clusters identified using the lower cutoff (0.018) yielded similar results to those identified using the higher cutoff (Table 3 and Supplementary Table S3). In the adjusted analysis, genotype 1a and seroconverter status were independently associated with clustering.

Factors associated with clustering in the Core-HVR1 and HVR1 regions were also analyzed and the results are comparable to those obtained using NS5B; however, some minor differences were observed dependent on the region and cutoff examined (Table 3). In a sensitivity analysis, factors associated with clustering were similar to those identified using the higher or lower cutoffs (Table 3).

4. Discussion

This study demonstrates the feasibility of using sequenced-based phylogenetics to identify recent HCV transmission clusters, which could support surveillance of population-level transmission dynamics. A convenience sample of sequential HCV RNA positive specimens from individual seroconverters permitted measurement of the expected intra-person HCV sequence evolution within 1 year. The maximum intra-person patristic distances were used as cutoffs to identify recent transmission clusters, and a higher cutoff was also applied to account for greater inter-person sequence variation that results from a change in host environments and immune pressures following transmission (Bull et al., 2011; Kuntzen et al., 2007; Mcallister et al., 1998; Merani et al., 2010). Using the lower and higher cutoffs, 63–92 unique individuals, were identified within clusters. Twenty-nine individuals in clusters were first-time positive testers with unknown infection duration and their identification within clusters indicates their involvement in a recent transmission event.

Individuals in clusters were more likely to have characteristics associated with incident infection in PWID (young age,

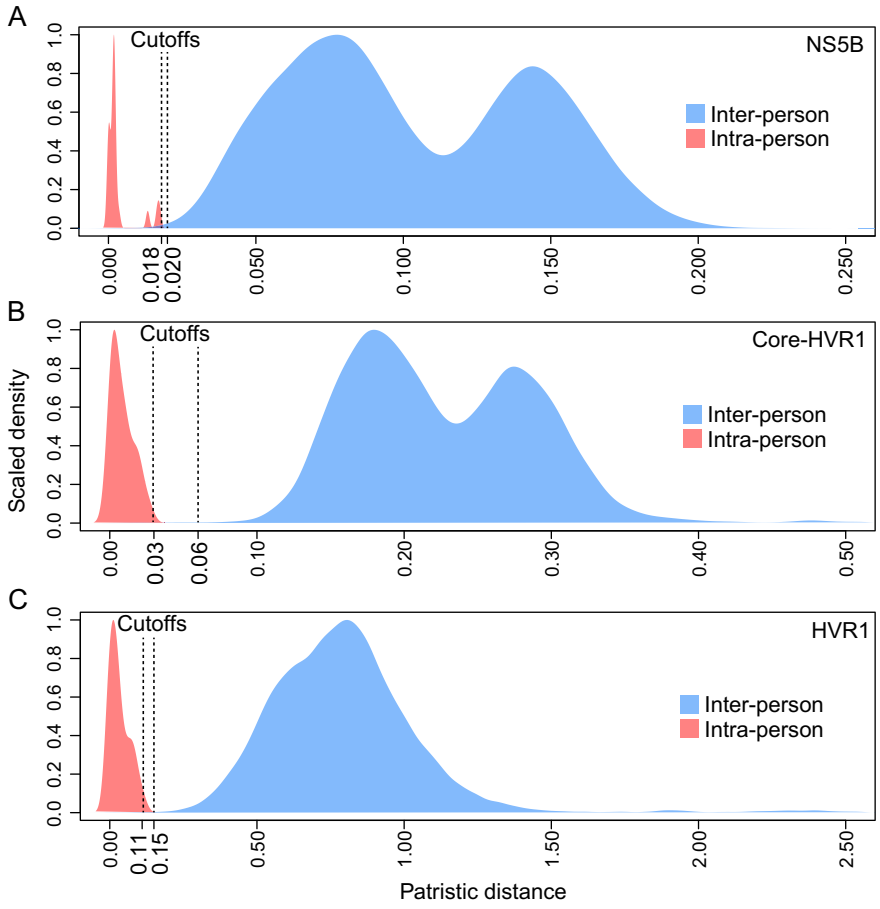

Fig. 2. Patristic distance frequency distributions. Frequency distribution of patristic distances between (A) 574 independent NS5B sequences obtained from different individuals and between 22 pairs of sequential sequences from 17 individuals (B) 396 Core-HVR1 sequences obtained from different individuals and between 20 pairs of sequential sequences from 18 individuals. (C) 445 independent HVR1 sequences obtained from different individuals and between 22 pairs of sequential sequences from 20 individuals. Blue = inter-person; red = intra-person. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seroconversion, Genotype 1a and 3a) (Jacka et al., 2014; Miller et al., 2002; Oliveira et al., 2009; Sacks-Davis et al., 2012) and were also more likely to have infection duration <1 year, supporting the hypothesis that individuals in clusters were involved in recent transmission events. In BC, >80% of seroconverters report injection drug use as their primary HCV acquisition risk factor. Consistent with these findings, a recent study by Jacka et al. (2014) demonstrated that individuals in clusters from a BC PWID cohort were more likely to be <40 years and to be seroconverters. These findings complement BC surveillance data, which shows that acute HCV infections are infrequent in baby boomers (Kuo et al., 2015) and substantiates a lower onward transmission risk in this birth cohort.

Three regions, the HVR1, Core-HVR1 and NS5B, were examined because of their different genetic evolutionary rates, which allow transmissions over different time scales to be examined. Evolution of HVR1 is not linear (Mcallister et al., 1998) and mutations accumulate rapidly (Dowd et al., 2009; Kuntzen et al., 2007; Liu et al., 2010) making this region useful for verifying person-to-person transmissions and for monitoring HCV genomic changes over short but not longer time periods (Escobar-Gutierrez et al., 2012; Weiner et al., 1993). Use of the highly conserved Core region combined with E1 and HVR1 increases the capacity to identify transmissions that may not be apparent with HVR1 alone, and as expected, a larger number of clusters were identified using Core-HVR1. The NS5B region evolves at an intermediate and more stable rate, which allows transmissions over longer time periods to be examined while still supporting identification of recent transmission events (Cavalheiro Nde et al., 2009; Hmaied et al., 2007). The NS5B amplicon used for this study was longer than that typically used (650 bp vs. 380 bp) (Jacka et al., 2013), which may have improved its ability to identify clusters.

The largest number of clusters was identified in NS5B and while many sequences were unavailable for comparison in the other regions, several NS5B clusters were also observed in HVR1 and/or Core-HVR1. Identification of clusters in two regions provides stronger support for transmission events. Some NS5B clusters had highly divergent Core-HVR1 or HVR1 sequences, suggesting no recent transmissions occurred between these individuals. In other NS5B clusters, the patristic distances between HVR1 and/or Core-HVR1 sequence pairs were only slightly above the cutoffs, suggesting that the cutoff in highly variable regions could be relaxed further to capture additional individuals within a transmission network. No clusters were identified between BC samples and the Los Alamos reference sequences, which supports the stringency of our method. Additional validation was provided by the use of confirmed transmission pairs, which were found to cluster in the NS5B region using the assigned cutoffs. It is important to note that various factors can influence the rate of HCV evolution over time (i.e. acute vs. chronic HCV infection, co-infection with HIV and immune status (Jabara et al., 2014; Merani et al., 2010; Ramachandran et al., 2011; Ray et al., 2005)), which will influence the patristic distance cutoffs and the identification of recent transmission clusters. Future work will further assess the utility of using

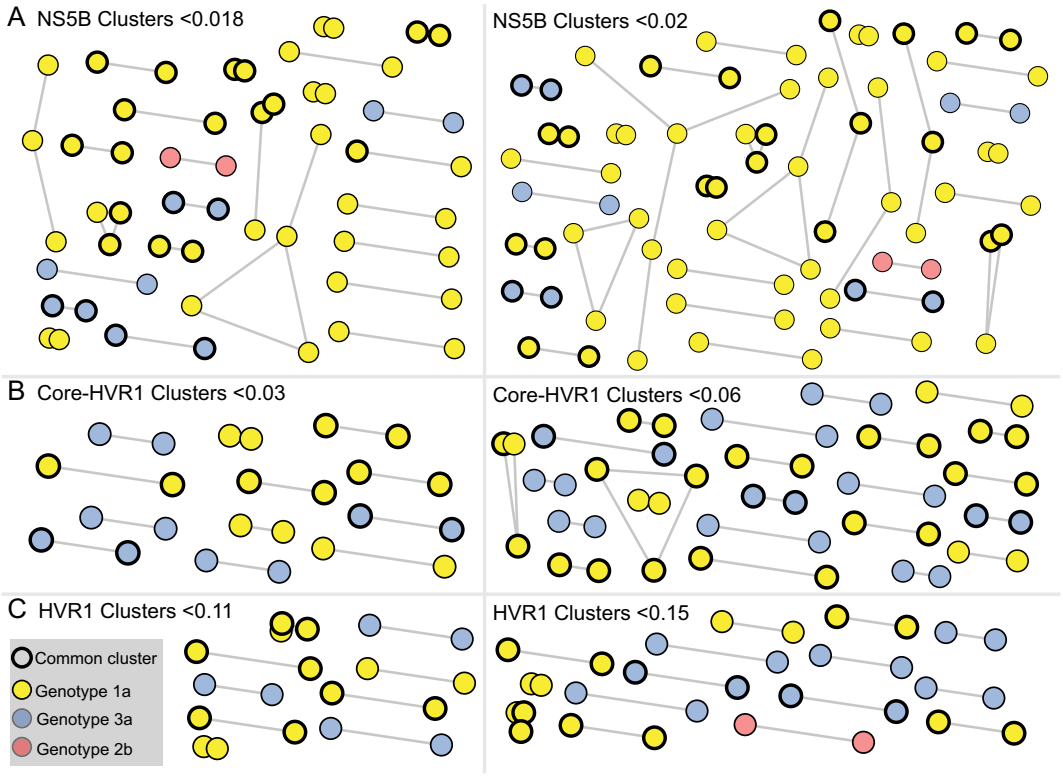

Fig. 3. Transmission clusters. (A) Inter-person (between different individuals) NS5B sequence clusters with patristic distances <0.018 (left) or <0.02 (right). (B) Inter-person Core-HVR1 sequence clusters (between different individuals) with patristic distances <0.03 (left) or <0.06 (right). (C) Inter-person HVR1 sequence clusters (between different individuals) with patristic distances <0.11 (left) or <0.15 (right). Nodes represent individual sequences and the connector length is proportional to the patristic distance between sequences. Common clusters (bold outline) are those that occur in both NS5B and Core-HVR1 or HVR1 alone.

Table 2
Number of clusters and individuals in clusters with various patristic distance cutoffs.

Cutoff	Total *n*[a]	*n* in clusters	% clustering	Number of clusters
NS5B				
0.018	574	55	10	25
0.020		73	13	31
0.022[b]		82	14	34
Core-HVR1				
0.03	415	24	6	12
0.06		48	12	23
0.09[b]		88	21	38
HVR1				
0.11	445	19	4	9
0.15		31	7	15
0.19[b]		45	10	20

[a] *n* = Number of individuals.
[b] Sensitivity analysis.

various regions and cutoffs for characterizing population level transmission dynamics.

A limitation to this study is that the serum samples used were collected in 2011 for the purposes of antibody screening, and were not stored under optimal conditions. For real-time population studies, these protocols would be applied to fresh serum samples within a day of collection or to blood samples containing EDTA to increase HCV RNA integrity, which should improve sequence yield. A further limitation to this study was the use of the midpoint between the last negative and first positive test date to estimate infection duration in seroconverters. For HIV, seroconverters identified through routine public health screening were more likely to have been infected closer to the time of the first positive test (Skar et al., 2013). Thus, individuals with long gaps between the last negative and first positive tests and an estimated HCV infection duration >1 year may have in fact been infected more recently. This likely explains, in part, the association between clustering and being a past seroconverter with infection duration >1 year. On the other hand, unlike with HIV, it has not been demonstrated that acute/early infections disproportionately drive HCV transmission. Many HCV infected individuals go undiagnosed for years or even decades (Rein et al., 2012; Shah et al., 2013), also HCV viral loads can remain high and stable throughout the course of infection, and the majority of HCV infected individuals remain untreated. This suggests that chronically infected individuals may be a significant source of onward HCV transmission and thus it is unsurprising that past seroconverters are also linked with transmission clusters.

The cluster analyses presented here cannot be directly extrapolated to create population incidence estimates given that this was a proof-of-principle study with a selection bias for seroconverters to maximize the identification of transmission clusters. In the future, these methods may be used, in combination with other strategies (i.e. tracking seroconverters and using diversity to estimate transmission timing (Montoya et al., 2015)) to obtain a comprehensive view of HCV incidence in BC. Every year >2000 individuals undergo HCV genotyping at the BC Centre for Disease Control and it would be feasible to apply routine sequencing and phylogenetics to these individuals given the declining cost of sequencing. Implementing this type of strategy could help to identify a subset of individuals with recent infection with no prior negative test available. It can also be used to identify clusters of individuals that are transmitting HCV and who may be targets for HCV prevention strategies.

This study supports the application of sequencing, phylogenetics, and the use of patristic distance cutoffs to identify recent transmission clusters between HCV infected individuals with unknown

Table 3
Multivariate regression analysis of factors associated with clustering in NS5B alone, Core-HVR1 alone, and HVR1 alone using multiple cutoffs.

	Adjusted Odds Ratio (95% CI)									
	NS5B			Core-HVR1			HVR1			
	0.018	0.02	0.022[a]	0.03	0.06	0.09[a]	0.11	0.15	0.19[a]
Characteristic									
Age	1.85 (0.98, 3.50)	**2.12 (1.21, 3.73)**	**2.28 (1.33, 3.93)**	1.27 (0.51, 3.19)	1.98 (1.01, 3.95)	**1.85 (1.07, 3.18)**	1.53 (0.55, 4.38)	1.16 (0.52, 2.63)	0.89 (0.43, 1.82)
Recent seroconverter	**3.72 (1.41, 9.47)**	**3.13 (1.29, 7.36)**	2.25 (0.94, 5.16)	**9.77 (2.53, 42.33)**	**6.54 (2.27, 19.77)**	**3.38 (1.47, 7.81)**	3.80 (0.80, 16.97)	**6.58 (1.71, 26.79)**	**6.74 (2.27, 20.15)**
Past seroconverter	**2.34 (1.20, 4.62)**	**2.19 (1.22, 3.97)**	1.60 (0.91, 2.81)	**4.33 (1.41, 16.17)**	**3.64 (1.57, 9.24)**	1.68 (0.92, 3.06)	2.53 (0.81, 8.76)	**4.83 (1.73, 15.79)**	**3.50 (1.55, 8.35)**
Genotype 1a	**4.29 (1.26, 26.9)**	**6.60 (1.98, 41.0)**	**8.11 (2.45, 50.2)**	N/A[b]	5.69 (1.13, 103.69)	**4.91 (1.70, 20.81)**	N/A[b]	1.34 (0.34, 8.85)	2.79 (0.78, 17.85)
Genotype 3a	3.79 (0.93, 25.6)	3.85 (0.95, 26.0)	4.28 (1.06, 28.8)	N/A	6.07 (1.12, 113.01)	**5.34 (1.70, 23.65)**	N/A	2.12 (0.51, 14.56)	2.37 (0.58, 16.02)

Abbreviations: AOR: Adjusted Odds Ratio, CI: Confidence Interval, SD: Standard Deviation.
Numbers in bold have a p-value < 0.05.
[a] Sensitivity analysis.
[b] N/A indicates the fitted probabilities numerically 0 or 1 occurred when the regression models included genotype.

infection histories. This technique, when applied at the population level and performed on an ongoing basis, may allow monitoring of HCV transmission networks and the changing dynamics of clusters over time, i.e. if new clusters arise from pre-existing clusters and if the number of clusters increases or decreases over time. This in turn could enable monitoring of transmission patterns in response to increased HCV screening and treatment uptake. It may also facilitate the tracking and evaluation of prevention interventions such as harm-reduction and treatment-as-prevention.

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Author contributions

ADO and MK designed this study with input from JJG, BJ, AFYP, JBJ, PRH, VM, YK and JM. ADO and VM performed all of the data analysis for this study with input from MK, JG, JBJ and AFYP. ADO, VM and IL performed all laboratory work for this study. The manuscript was written by ADO with assistance from MK and DC and with input from all contributing authors.

Potential conflicts of interest

MK has received grant funding via his institution from Roche Molecular Systems, Boehringer Ingelheim, Merck, Siemens Healthcare Diagnostics and Hologic Inc. JG is a consultant/advisor and has received research grants from Abbvie, Bristol Myers Squibb, Gilead and Merck. PRH has received grants from, served as an ad hoc advisor to, or spoken at various events sponsored by: Pfizer, Glaxo-SmithKline, Abbott, Merck, Tobira Therapeutics, Virco and Quest Diagnostics and served as a consultant for ViiV Health Care, Tobira Therapeutics, Selah Genomics Inc, and Quest Diagnostics. He holds stock in Merck, Illumina, Gilead and EKF Diagnostics. JM has received grants from Abbott, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck and ViiV Healthcare. All other authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.04.017.

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